Journal of Cellular Biochemistry 70:563–572 (1998)

Stimulation of Heparan Sulfate Proteoglycan Synthesis and Secretion During G_1 Phase Induced by Growth Factors and PMA

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Abstract Fetal calf serum (FCS) and PMA (phorbol 12-myristate-13-acetate) specifically stimulate the synthesis of heparan sulfate proteoglycan in endothelial cells. Staurosporine and n-butanol, kinase inhibitors, abolish the PMA effect. Forskolin and 8-bromo adenosine 3':5'-cyclic monophosphate, activators of, respectively, adenylate cyclase and protein kinase A cannot reproduce the PMA effect. The kinetics of cell entry into S phase of the endothelial cells was determined by DNA synthesis ([^{3}H]-thymidine and Br-dU incorporation), and flow cytometry. The mitogenic effect of fetal calf serum is abolished by PMA. Also, PMA pre-treatment inhibits the enhanced synthesis of heparan sulfate proteoglycan after a second PMA exposure. Remarkably, the stimulation of heparan sulfate proteoglycan synthesis by fetal calf serum and PMA seems to be mainly restricted to G_1 phase. Therefore fetal calf serum and PMA cause an enhanced synthesis of heparan sulfate proteoglycan, and PMA causes a cell cycle block at G_1 phase. J. Cell. Biochem. 70:563–572, 1998.

Key words: heparan sulfate and growth factors; heparan sulfate and phorbol ester; heparan sulfate and cell cycle; proteoglycans and cell cycle; cell cycle; phorbol ester and heparan sulfate; heparan sulfate and PKC

Heparan sulfate proteoglycans (HSPG) are present at the cell surface of mammalian cells in culture and in most vertebrate and invertebrate tissues [Dietrich, 1984; Dietrich and Montes de Oca, 1970; Dietrich and Nader, 1974; Gallagher et al., 1986; Kraemer, 1971; Nader et al., 1984; Poole, 1986; Rodén, 1980; Ruoslahti, 1989]. They are composed of alternating units of glucosamine and uronic acid (glucuronic or iduronic) and the hexosamine is either N-acetylated or N-sulfated and/or 6-sulfated [Dietrich et al., 1983; 1998; Fransson, 1989; Gallagher and Lyon, 1989].

Abbreviations: 8 BrcAMP, 8-bromo adenosine 3′:5′-cyclic monophosphate; FCS, fetal calf serum; HSPG, heparan sulfate proteoglycan; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate.

Contract grant sponsors: FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), FINEP (Financiadora de Estudos e Projetos), and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil.

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Received 15 January 1998; Accepted 26 March 1998

Some of the most basic biological phenomena depend on precise chemical interactions between components of cell membrane and extracellular matrix [Ruoslahti, 1989]. The biological relevance of potentially specific interactions of the glycosaminoglycan moiety of the proteoglycans with other glycosaminoglycans and different molecules of cell membrane and matrix was realised long ago [Jackson et al., 1991; Yamada, 1983]. In fact a number of reports provided experimental evidences suggesting that heparan sulfate chains play a role in cellular recognition, cellular adhesion, and growth control [Fransson, 1989; Dietrich and Armelin, 1978; Dietrich et al., 1977, 1982; Ishihara et al., 1986; Kraemer and Tobey, 1972]. In addition, several papers describe high affinity association of heparin-like molecules with growth factors [Castellot et al., 1984; Gospodarowicz et al., 1984; Klagsbrun et al., 1987; Lobb et al., 1986; Thompson et al., 1988; Yayon et al., 1991], implying that heparan sulfate growth effects are likely to be mediated by growth factors [Gallagher et al., 1986; Gambarini et al., 1993; Yayon et al., 1991]. On the other hand, experi-

mental indication of direct correlation between cell cycle events and proteoglycan metabolism are still scarce [Dietrich, 1984; Ruoslahti, 1989].

The control of cell cycle is mostly exerted by growth factors and hormones during the G_1 phase [Pardee, 1989]. Phorbol 12-myristate-13-acetate (PMA) is a tumor promoting phorbol ester that activates protein kinase C (PKC) [Backer and King, 1991; Castagna et al., 1982]. PMA mimics the mitogenic actions of growth factors inducing the early response genes required for G_1 phase cell transversing and S phase entry [Herschman, 1991]. These results led to the largely accepted notion that PKC is an essential component in the signal transduction routes activated by growth factors receptors. However, recent studies from the laboratory of one of us [Faria and Armelin, 1996] have shown that PMA, actually, displays a dual effect in $G_0 \rightarrow G_1 \rightarrow S$ transition: it is stimulatory in early G_1 ($G_0 \rightarrow G_1$ transition), in agreement with its mitogenic activity. But in late G_1 ($G_1 \rightarrow S$ transition) PMA is inhibitory, blocking S phase entry. Both, stimulatory and inhibitory PMA effects seem to be mediated by PKC activation [Faria and Armelin, 1996]. Therefore, this novel PMA inhibitory effect is, first, uncovering a G_1 checkpoint at which cells are sensitive to a PKC-mediated growth block and, second, is emphasizing the usefulness of PMA as a versatile tool to probe into the organization and regulatory mechanisms of the $G_0 \rightarrow G_1 \rightarrow S$ transition of cell cycle.

Our interest in this study is to search for correlations between proteoglycan synthesis and cell proliferation. Since in previous work we have defined the structure of heparan sulfate from an endothelial cell line [Nader et al., 1987, 1989], we have decided to use these cells as a working model for the present studies. We show here that growth factors, present in fetal calf serum, and PMA cause a specific stimulation of heparan sulfate proteoglycan synthesis. On the other hand, PMA blocks $G_1 \rightarrow S$ transition of cell cycle when cells are induced to proliferate by FCS. We also show that the response of these cells to PMA regarding HSPG synthesis is mediated by PKC activation. A preliminary communication of part of these findings has appeared [Porcionatto et al., 1994].

MATERIALS AND METHODS

Substrates, Enzymes, and Materials

Heparan sulfate from bovine pancreas prepared by quaternary amine precipitation [Cifonelli and Dorfman, 1960] was a gift from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy) and heparin from bovine lung from Dr. L.L.Coleman, UpJohn Co. (Kalamazoo, MI). Chondroitin 4- and 6-sulfates, dermatan sulfate, chondroitinases AC, and ABC were purchased from Seikagaku Kogyo (Tokyo, Japan). Heparinase, heparitinases I and II, disaccharide sulfoesterase, and glycuronidase were prepared from induced *Flavobacterium heparinum* cells by methods described previously [Dietrich et al., 1973; Nader et al., 1990]. Ethylenediamine (1,2-diaminoethane) and propylenediamine (1,3-diaminopropane) were purchased from Aldrich Co. (Milwaukee, WI), staurosporine from Boehringer Mannheim Biochemica (Mannheim, Germany), and phorbol 12-myristate-13-acetate (PMA), forskolin, calcium ionophore (A23187), 8-bromo adenosine 3′:5′-cyclic monophosphate, Br-dU, anti-Br-dU antibody, DAPI, FITC-conjugated mouse IgG secondary antibody, Triton X-100, Nonidet P-40 from Sigma Chemical Co. (St. Louis, MI). Superase (protease from sporobacillus) from Chas Pfizer Co. (New York, NY). D-[1,6-^{3}H-N)] glucosamine hydrochloride (30 Ci/mmol), [^{14}C]-amino-acids mixture (50 µCi/ml) and [^{3}H]-methyl thymidine (85 Ci/mmol) were purchased from Amersham Co. (Buckinghamshire, UK), carrier free [^{35}S]-sulfuric acid and [^{32}P]-phosphoric acid from IPEN (São Paulo, SP, Brazil).

Cell Culture

An endothelial cell line derived from rabbit aorta [Buonassisi and Venter, 1976] was maintained in F-12 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Cultilab, Campinas, SP, Brazil) at 37°C, 2.5% CO_2. Quiescent cultures (3×10^5 cells/plate) were obtained by maintaining the cells in serum-free medium for 24 h at 37°C, 2.5% CO_2 before treatment with the drugs.

[^{3}H]-Thymidine Incorporation, Br-dU Incorporation, and Flow Cytometry

For the incorporation of [^{3}H]-thymidine, quiescent cultures were incubated with [^{3}H]-methyl thymidine (0.25 µCi/ml) for the times indicated in the experiments. The cells were then washed with 5% TCA and the amount of radioactivity incorporated into cells determined by counting the cell extracts obtained after incubation with 0.5 N NaOH for 1 h at 37°C as described [Armelin, 1973].

For Br-dU incorporation, cells were seeded on coverslips in F12 medium with 10% FCS. After 24 h cells were serum starved for 24 h. Cells were treated with PMA or FCS and then incubated with 100 µM Br-dU. Cells were fixed with cold methanol for 10 min and stored. Coverslips with fixed cells were incubated with 1.5N HCl for 30 min, inverted onto a 40 µl drop of mouse anti-Br-dU antibody, and incubated for 30 min at room temperature. Coverslips were washed in phosphate-buffered saline and immune complexes were diluted with a FITC-conjugated mouse IgG secondary antibody. The coverslips were incubated with DAPI (5 µg/ml) for 20 min to stain total nuclei and then examined under a Nikon Fluorophot microscope using two excitation filters: UV 330–380 nm to visualize DAPI-stained total nuclei and IF 420–490 nm to visualize the Br-dU labelled nuclei stained with FITC. Around 500–600 nuclei were counted per coverslip.

For the cytometric DNA analysis, quiescent cultures were stimulated with 10% FCS for different periods of time, in the presence and absence of PMA (100 ng/ml). The cells were dettached from the culture dish using trypsin and submitted to the flow cytometric analysis as previously described [Vindeløv and Christensen, 1990].

Labelling of Cells and Extraction of Proteoglycans and Glycosaminoglycans

Proteoglycans synthesized by the cells were metabolically labelled with either [^{35}S]-inorganic sulfate (150 µCi/ml), [^{3}H]-glucosamine (50 µCi/ml), or [^{14}C]-amino acids (50 µCi/ml). The radioactivity was expressed as cpm/µg of cell protein. Quiescent cultures were incubated for different periods of time with PMA, 10% FCS, or other additions as indicated. At the end of the incubation, the culture medium was removed and the cells were washed twice with F-12 medium. The cells were scrapped from the dish with 3.5 M urea in 25 mM Tris-HCl pH 7.8. Both cells and medium were precipitated in the cold with five volumes of ethanol in the presence of 100 µg of carrier heparan sulfate, dermatan sulfate, and chondroitin sulfate. The radioactive glycosaminoglycan free chains were prepared from the cells and culture medium by incubation with 0.1 mg of Superase for 4 h at 60°C in the presence of 0.8 M NaCl, pH 8.0 in a final volume of 200 µl. After incubation, the mixture was heated for 10 min at 100°C (to inactivate the proteolytic enzyme) and the glycosaminoglycans were precipitated with two volumes of methanol at −20°C.

Identification and Quantitation of Glycosaminoglycans and Proteoglycans

The proteoglycans and sulfated glycosaminoglycans were identified and quantitated by a combination of agarose gel electrophoresis and enzymatic degradation as previously described and the results presented are mean of, at least, three determinations [Nader et al., 1987, 1989]. For quantitation, the radioactive bands were scrapped off the agarose gels (after fixation, drying, and staining) dissolved in 1 ml of 1 M HCl, heated at 100°C for 5 min, and counted in 10 ml of Hyonic Fluor (Packard Inst., Meriden, CT) in a liquid scintillation spectrometer. Cell protein was determined as follows: after incubation, the dishes containing the cells were washed three times with 2 ml of serum-free F12 medium and the cells dispersed in 500 µl of a 25 mM solution of Tris-HCl buffer, pH 7.8 containing 3.5 M urea. Protein was determined in 10 µl aliquots by the coomassie blue assay [Spector, 1978].

A typical incubation mixture contained 0.1U of enzymes, 10–50 $\times 10^3$ cpm of sulfated glycosaminoglycans with 50 µg of carrier sulfated glycosaminoglycans and other additions as indicated in 0.05 M ethylenediamine-acetate buffer, pH 7.0 in a final volume of 30 µl. The incubation mixtures were spotted in Whatman nr 1 paper and subjected to chromatography in isobutyric acid: 1.25 M NH_3, 5/3.6, v/v for 24 h. The unsaturated products formed from the carrier compounds were detected by short wave UV lamp. The radioactive [^{35}S]-labeled products were located by exposure of the chromatograms to Kodak X-ray film (SB-5) for 3 to 15 days. For the [^{3}H]-labeled heparan sulfate the degradation products were located using the migration of the carrier. They were quantitated by counting the paper containing the radioactive compounds in 0.5% PPO in toluene in a liquid scintillation spectrometer. When the radioactivity of the disaccharides was measured using [^{35}S]-sulfate isotope, the actual amount of each compound was calculated by dividing the radioactivity incorporated into the individual species by the number of sulfate residues present in the molecule. The different radioactive enzymatic products obtained from the heparan sulfate upon the action of the enzymes were identified by their chromatographic migrations in two different solvents as well as their electro-

phoretic mobilities [Nader et al., 1987, 1989] using standard disaccharides prepared from heparin and heparan sulfate whose structures have been determined by chemical analyses and nuclear magnetic resonance [Nader et al., 1990]. Also, the structure of the radioactive disaccharides were further confirmed by degradation with specific sulfatases and glycuronidase as previously described [Nader et al., 1987].

RESULTS

PMA Stimulates the Synthesis and Secretion of Heparan Sulfate

In order to probe if the PKC pathway would affect HS synthesis we used the PKC activator, PMA. Figure 1A displays a dose-response curve for PMA indicating that the uptake of [^{35}S]-sulfate in HS of cell extract doubles at very low PMA concentration whereas the rate of HS secretion to the medium increases up to 100ng/ml of PMA, leveling off thereafter. The synthesis of the other major class of glycosaminoglycan, namely chondroitin sulfate, as well as the sulfated glycoproteins found in this cell line, were not affected by PMA (not shown). Figure 1B shows that PMA produces a rapid increase in HS synthesis (between 2 and 8 h) leveling off from 10 to 24 h. The lag between addition of PMA and the onset of HS synthesis increase is around 30–45 min (Fig. 1C). A pulse-chase experiment showed that the rate of secretion of heparan sulfate is not affected by the addition of PMA (data not shown).

PMA in Fact Stimulates the Synthesis of Both Protein Core and Polysaccharide Moiety of Heparan Sulfate Proteoglycan

Quiescent endothelial cells were exposed to PMA (100ng/ml) in the presence of [^{35}S]-sulfate and the proteoglycans present in the cell extract and secreted to the medium were analyzed by agarose gel electrophoresis. A substantial enhancement of HSPG is observed in both cell extract and medium. The enhancement was also observed when cells were labelled with a mixture of [^{14}C]-amino acids indicating that the synthesis of both, core protein and glycosaminoglycan chains, are stimulated by PMA. Table 1 shows the ratio of [^{35}S]-sulfate, [^{3}H]-glucosamine, and [^{14}C]-amino acids uptake into HSPG liberated into the medium, in function of PMA concentration. The results indicate that the ratio of sulfate/glucosamine/amino acids is constant irrespective of PMA concentration suggesting that the heparan sulfate proteoglycan species whose synthesis is stimulated by PMA is invariant. This conclusion is further supported by the data of Table 2 showing that the disaccharide composition of heparan sulfate

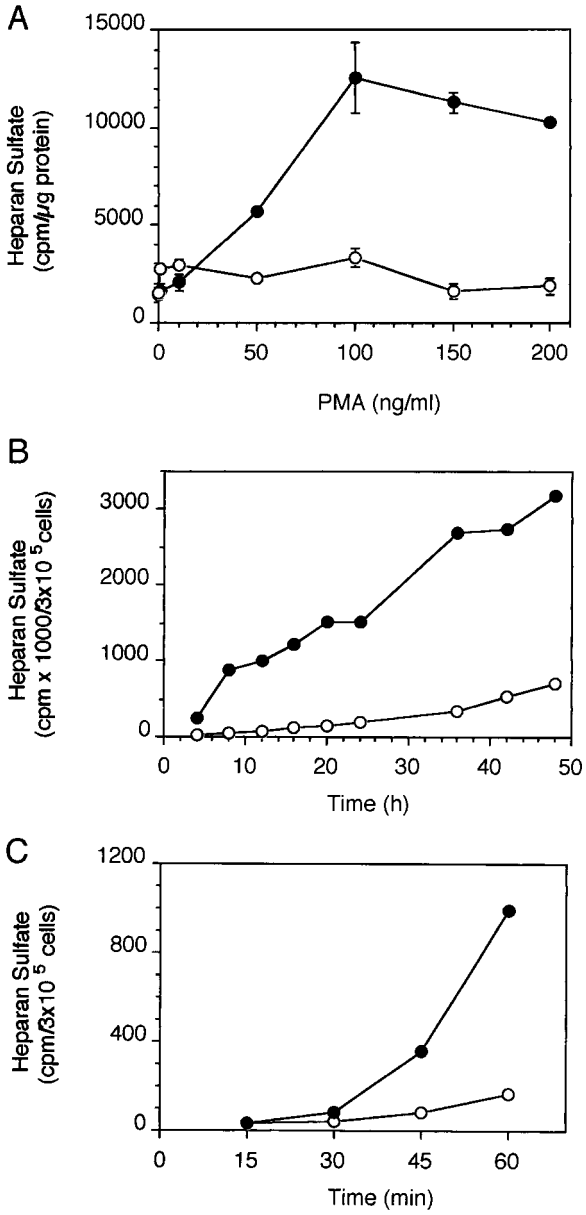

Fig. 1. Time course and concentration dependence of HSPG synthesis by endothelial cells in the presence of PMA. **A:** The cells were metabolically labeled with [^{35}S]-sulfate for 18 h, at 37°C, in the absence and presence of different concentrations of PMA (0–200 ng/ml). The amount of HSPG synthesized by the cells (O-O) as well as secreted to the medium (O-O) was measured. **B,C:** Endothelial cells were exposed to [^{35}S]-sulfate in the absence (O-O) and presence of PMA (100 ng/ml) (●-●) for different periods of time, as indicated, and the amount of HSPG secreted to the medium was determined.

chains is the same for control and PMA-treated cells. Furthermore, the M.W. of the heparan sulfate chains was not affected by treatment with PMA (15–50 kDa, modal M.W. 35 kDa), as well as the M.W. of the intact HSPG.

The PMA Effect, Stimulating Heparan Sulfate Proteoglycan Synthesis, Is Likely to be Mediated by PKC Activation

A 18 h PMA pretreatment renders cells resistant to a second PMA treatment (Fig. 2), suggesting that the synthesis of HSPG is downregulated as it has been shown for PKC activation [Nishizuka, 1992]. Also, PMA causes a rapid phosphorilation of an 80 kDa protein (not shown) that is known to be phosphorilated by PKC in fibroblast cells [Rozengurt et al., 1983]. These results suggest that PMA activates PKC in the endothelial cells. Besides, staurosporine and n-butanol, inhibitors of protein kinases, abolish the PMA effect (Table 3). On the other hand, forskolin, an activator of adenylate cyclase, and 8BrcAMP, an activator of PKA, do not mimic the PMA effect. Therefore, the kinase inhibitors block the effect of PMA whereas, activators of cAMP/PKA pathway do not stimulate heparan sulfate synthesis, strongly suggesting that PKC is the mediator of this effect.

TABLE I. Incorporation of [^{35}S]-Sulfate, [^{3}H]-Glucosamine and [^{14}C]-Amino Acids on Heparan Sulfate Proteoglycan Synthesized in the Presence of Different Concentrations of PMA

	Medium heparan sulfate proteoglycan (cpm/3 × 10^5 cells)			
PMA (ng/ml)	Sulfate × 10^3	Glucosamine	Amino acids	Ratio Sulf/GlcN/AA[a]
0	379	5,760	1,450	1/15.1/3.8
10	680	9,300	—	1/13.6
50	1663	21,500	—	1/12.9
100	3304	45,400	11,150	1/13.7/3.4

[a]Sulf, sulfate; GlcN, glucosamine; AA, amino acids.

TABLE II. Disaccharide Composition of Heparan Sulfate Synthesized by Endothelial Cells in the Absence and Presence of PMA*

	Heparan sulfate products (%)			
	Medium		Cells	
Disaccharide	Control	PMA	Control	PMA
ΔU,2S-GlcNS,6S	9.3	9.4	12.8	10.1
ΔU,2S-GlcNS + ΔU-GlcNS,6S	27.4	33.0	30.9	28.2
ΔU-GlcNS	54.3	52.7	50.4	50.8
ΔU-GlcNAc,6S	9.0	6.9	7.8	10.8

*ΔU,2S-GlcNS,6S: O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1-4)-2-sulfamino-D-glucose 6-sulfate; ΔU-GlcNS,6S: O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-sulfamino-D-glucose 6-sulfate; ΔU,2S-GlcNS: O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1-4)-2-sulfamino-D-glucose; ΔU-GlcNS: O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-sulfamino-D-glucose; ΔU-GlcNAc,6S: O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1-4)-2-acetamido-D-glucose 6-sulfate.

PMA Blocks $G_0/G_1 \rightarrow S$ Transition Promoted by FCS

Cell DNA content analysed by flow cytometry shows that endothelial cells, growth arrested by serum deprivation, are stimulated to enter S phase by FCS refeeding (Table 4). This growth response to FCS is mostly abolished by PMA. This inhibitory effect of PMA is confirmed by [^{3}H]-thymidine uptake into DNA as shown by the dose-response curve of Figure 3 indicating that 10 ng/ml PMA is already sufficient to cause

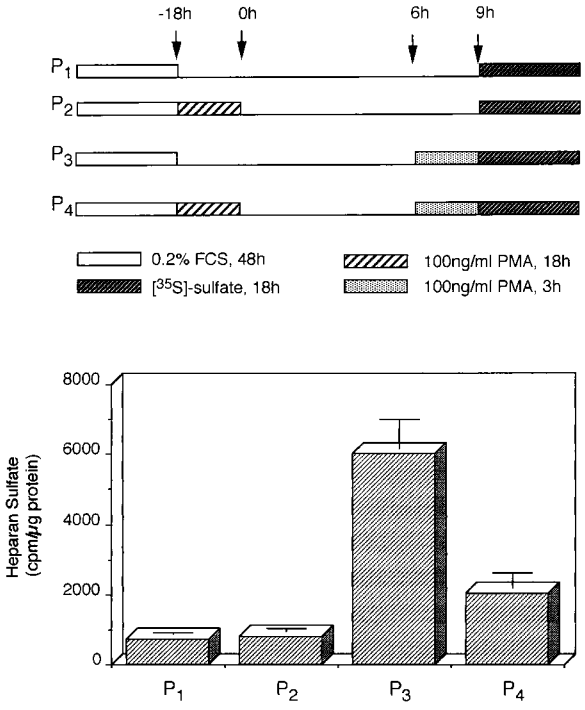

Fig. 2. Down-regulation of HSPG synthesis. Quiescent endothelial cells were submitted to two pulses (P_4) or one pulse of 100 ng/ml PMA (P_2 and P_3), or none (P_1) and labeled with [^{35}S]-sulfate as indicated in the upper panel. The amount of HSPG secreted to the culture medium is shown in the lower panel. The values are mean of three determinations.

TABLE III. Effect of Different Compounds on the Synthesis of Heparan Sulfate Proteoglycan by Endothelial Cells in Culture

Compound	Medium HSPG (cpm/µg cell protein)	Ratio (control/ treated)
None	401	1.0
PMA (100 ng/ml)	2358	12.3
Staurosporine (10 µg)	425	1.1
PMA (100 ng/ml) + Staurosporine (10 µg)	536	1.3
n-Butanol (2.5% v/v)	398	1.0
n-Butanol (2.5% v/v) + PMA (100 ng/ml)	1259	3.1
Forskolin		
10 µM	508	1.3
20 µM	438	1.1
8-Br-cAMP		
5.0 mM	454	1.1
2.5 mM	502	1.3

75% inhibition of DNA synthesis. Increase to 100 ng/ml of PMA did not change the inhibition of [^{3}H]-thymidine incorporation. These results were confirmed by Br-dU incorporation into control and treated cells (Table 5).

PMA Induction of Heparan Sulfate Synthesis Correlates With PMA Cell Cycle Block at G_1 Phase

Cells were stimulated to proliferate by the addition of 10% FCS at time zero and, then, periodically treated or not with PMA (100 ng/ml) in the presence of [^{35}S]-sulfate for 4 h, starting at time 2 h. Radioactivity incorporated into the HSPG secreted to the medium was determined and the result plotted at the point, in the time axis, correspondent to the middle of the respective incorporation period (Fig. 4). The wave of HSPG stimulated synthesis is mainly restricted to G_1 phase, picking at 4 h; remarkably between 14 and 18 h, when percentage of S phase cells is maximal (see Table 4), [^{35}S]-sulfate incorporated into HSPG is minimal (Fig. 4). Therefore, PMA causes a period of HSPG synthesis stimulation that preceeds S phase and, at the same time blocks the cell entry into S phase, abolishing the mitogenic effect of FCS refeeding.

DISCUSSION

PKC has a crucial role in signal transduction for a variety of biologically active substances, such as growth factors and hormones, whose activities balance cellular proliferation and differentiation [Nishizuka, 1992]. On the other hand, PMA was first known for its tumor promoting activity [Boutwell, 1974], and only later on, recognized as a strong and specific activator of PKC [Backer and King, 1991; Castagna et al., 1982], mimicking the natural modulator of this enzyme, i.e., dyacylglicerol [Berridge, 1993; Nishizuka, 1984]. Thus, presently, many biological effects are attributed to its ability to activate a series of isoforms of PKC.

We now report that PMA specifically stimulates the synthesis of heparan sulfate proteoglycan of endothelial cells, an effect that is likely to be mediated by PKC activation. Staurosporine and n-butanol, two kinase inhibitors [Aguirre Ghiso et al., 1996; Courage et al., 1995; Ding and Badwey, 1994] abolish the PMA effect whereas activation of cAMP/PKA pathway by both, forskolin and/or 8BrcAMP, is not effective to trigger stimulation of HSPG synthesis. Furthermore, 18 h PMA pretreatment, that presumably downregulates PKC [Nishizuka, 1992], renders endothelial cells more resistant to a second PMA treatment.

Stimulation of the cells to proliferate induced by growth factors present in FCS also stimulate the secretion of HSPG to the culture medium, showing a physiologic response of the cells. The most remarkable aspect of our results, however, is the possible correlation between the stimulation of HSPG synthesis and a block of G_1 phase traversing, both triggered by PMA. This observation is suggesting a coordinate cell response, elicited by PMA, that was not realized before, which links a cell cycle checkpoint with the metabolism of cell surface proteoglycans. Also, another curious finding is the synergistic effect of FCS and PMA in a lower dosis (10 ng/ml), indicating that there are two distinct pathways involved in the control of HSPG synthesis.

In spite of the largely accepted notion that PKC is important in signal transduction mechanisms, it is still obscure how the isoforms of this enzyme operate. Particularly, it is unclear how PKC is located in the regulation circuitry that controls the cell cycle. Results from the last 10 years have led to the proposal that the products of proto-oncogenes and tumor supressor genes (anti-oncogenes) are organized into two separated circuits that regulate $G_0 \rightarrow G_1 \rightarrow S$ transi-

TABLE IV. Effect of PMA on Endothelial Cell Cycle

Time after stimulation (hours)[a]	% of cells in each phase of the cell cycle					
	Control			PMA (100 ng/ml)		
	$G_0 \rightarrow G_1$	S	$G_2 \rightarrow M$	$G_0 \rightarrow G_1$	S	$G_2 \rightarrow M$
0	76.3	8.4	15.3	76.3	8.4	15.3
8	67.2	12.1	20.8	72.9	8.4	18.7
14	53.0	23.6	23.5	72.0	10.5	17.5
18	54.7	24.4	20.8	77.7	9.4	12.9
20	62.3	16.2	21.5	74.8	12.7	12.4

[a]Quiescent endothelial cells were stimulated with 10% FCS in the absence and presence of PMA (100 ng/ml) and cultures were harvested at various times after stimulation for flow cytometric analysis of DNA content.

TABLE V. Effect of PMA on the Incorporation of Br-dU by Endothelial Cells

Treatment	% of cells labeled with Br-dU[a]
Serum-free medium	19.1
PMA (10 ng/ml; 2 h)	4.4
FCS (10%; 2 h)	51.6
PMA (10 ng/ml; 12 h)	13.5
FCS (10%; 12 h)	77.3

[a]Ratio between number of Br-dU-labeled nuclei and number of DAPI-labeled nuclei.

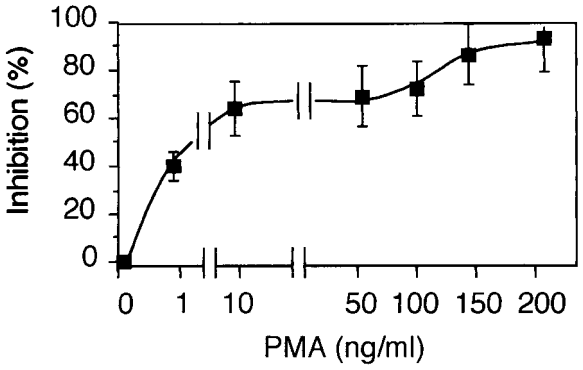

Fig. 3. Effect of PMA on DNA synthesis. Quiescent endothelial cells were stimulated with 10% FCS in the presence of different concentrations of PMA (0–200 ng/ml). The incorporation of [^{3}H]-thymidine was measured as described.

tion [Bishop, 1991; Herschman, 1991; Scherr, 1994]. Proto-oncogenes are concentrated in the regulatory pathway activated by growth factor receptors that stimulates cells to exit G_0 towards G_1. PMA, via PKC, also activates the proto-oncoproteins pathway, whose recognized end-effect is to modulate gene transcription. This scheme can explain PMA's mitogenic action. On the other hand, the anti-oncoproteins (Rb, p53, p21, p16) are located in the cyclin dependent kinases (CDKs) circuit that regulates the $G_1 \rightarrow S$ transition [Moreno and Nurse, 1994; Scherr, 1994]. The end result of CDKs activation is Rb phosphorilation, that also allows modulation of gene transcription. We hypothesize elsewhere [Faria and Armelin, 1996] that the PMA block of G_1 cell traversing is due to activation of a PKC isoform that inhibits CDKs activation, thus interrupting the phosphorilation cascade that leads to gene transcription modulation. Therefore, the dual effects of PMA in $G_0 \rightarrow G_1 \rightarrow S$ transition are both being considered in links with the two main pathways that regulate the gene transcription supposedly relevant to cell cycle control. Curiously, the mapping of the syndecans (heparan sulfate proteoglycans) genes has shown a linkage with members of the *myc* gene family [Spring et al., 1994]. Also, in vitro experiments have demonstrated that PKC is able to phosphorylate two of the four syndecan cytoplasmic domains [Prasthofer et al., 1995].

The novelty in the results of this paper is the emergence of a promising picture that connects regulatory circuits that modulate gene transcription with cell surface proteoglycans metabolic pathways in a coordinate fashion to control cell cycle. Results from several laboratories have been reported describing PMA effects on cell surface proteoglycans of chondrocytes [Lowe et al., 1978; Bouakka et al., 1988; Hoffman et al., 1993], human fibroblasts [Suzuki et al., 1995], and eritroleukemia cells [Grässel et al., 1995]. Hopefully, we are approaching the moment that will be possible to establish a unifying picture that coherently will display the links between cell surface proteoglycan metabolism and intracellular regulatory circuitry that maintain the homeostasis of proliferation and differentiation.

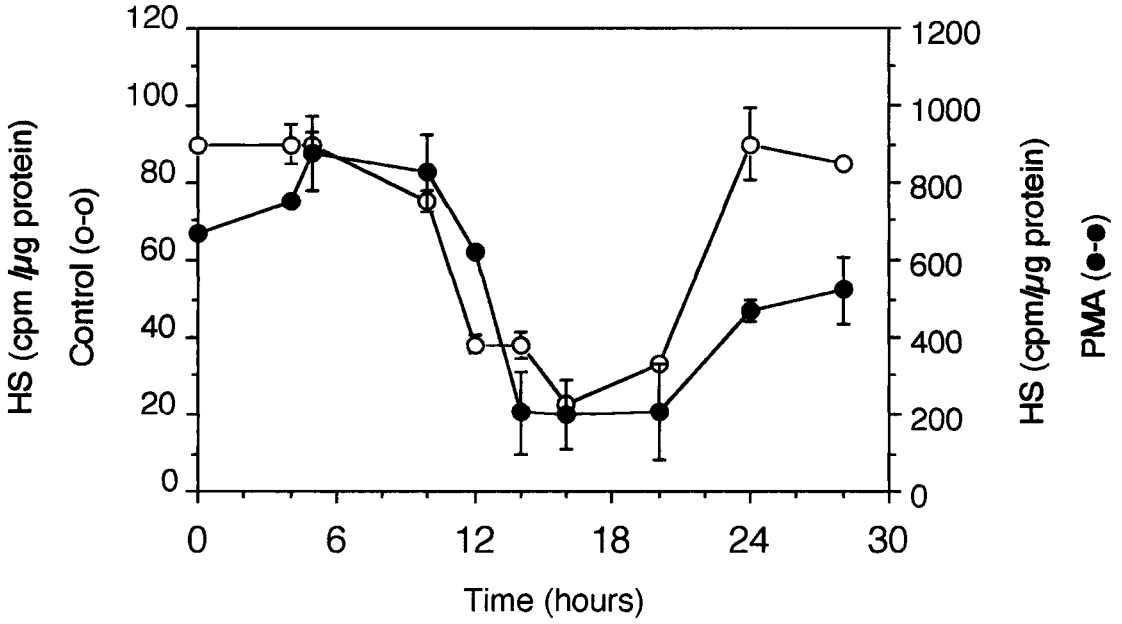

Fig. 4. Effect of PMA on the synthesis of HSPG secreted to the culture medium in different phases of the cell cycle. Quiescent endothelial cells were stimulated to proliferate by the addition of 10% FCS. After different periods of time, [^{35}S]-sulfate was added to the culture medium during 4 h, in the absence (O-O) and presence of 100 ng/ml PMA (●-●), and the amount of HSPG secreted to the medium was measured .

ACKNOWLEDGMENTS

We thank Dr. M. Brentani from Faculdade de Medicina, USP, São Paulo, Brazil, for the flow cytometric analysis. This work was aided by grants from FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), FINEP (Financiadora de Estudos e Projetos), and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil.

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